Attorney Docket No. 1033172-000001 IFW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	
Application No.: 09/319,736	)	Group Art Unit: 1643
	)	
Filed: August 2, 1999	)	Examiner: KAREN A CANELLA
	)	
For: THERAPEUTIC APPLICATIONS OF ANTIGENS OR EPITOPES ASSOCIATED WITH IMPAIRED CELLULAR PEPTIDE PROCESSING, E.G. EXPRESSED ON RMA-S CELLS TRANSFECTED WITH A B7-1 GENE	)	Confirmation No.: 3510

SUBMISSION OF CERTIFIED COPY OF PRIORITY DOCUMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The benefit of the filing date of the following priority foreign application in the following foreign country is hereby requested, and the right of priority provided in 35 U.S.C. § 119 is hereby claimed.

Country:	Sweden
Patent Application No.:	9604581-0
Filed:	December 12, 1996

In support of this claim, enclosed is a certified copy of said foreign application. Said prior foreign application is referred to in the oath or declaration and/or the Application Data Sheet. Acknowledgement of receipt of this certified copy is requested.

Respectfully submitted,

BUCHANAN INGERSOLL PC

Date: February 23, 2006

By: _____
Christopher L. North, Ph.D.
Registration No. 50,433

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

Intyg
Certificate

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

(71) *Sökande* *Karolinska Innovation AB, Solna SE*
Applicant (s)

(21) *Patentansökningsnummer* *9604581-0*
Patent application number

(86) *Ingivningsdatum* *1996-12-12*
Date of filing

Stockholm, 2006-02-14

För Patent- och registreringsverket
For the Patent- and Registration Office

Hjördis Segerlund

Avgift
Fee *170:-*

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

Karolinska Innovation AB

An agent against cancer and virus infections

The present invention relates to use of substances that can induce expression of antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens for preparation of pharmaceuticals, pharmaceutical compositions or vaccines, that stimulate specific T cell mediated immune responses against cancer and virus infected cells. It also relates to mammalian cells that have been manipulated to express antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens and to lymphoid cells activated against such MHC class I dependent structures for the same purpose. Furthermore, processes for such manipulation of mammalian cells and kits for use in such manipulations are covered. According to the present invention, the ultimate purpose of the products or processes above is the treatment of cancers and virus infections.

Background of the invention

The immune system recognizes material foreign to the body (so called antigen) and eliminates this material. An important part of the immune system is composed of $CD8^+$ cytotoxic T cells or T-lymphocytes (CTL), which recognize foreign and sick cells, e.g. in virus infections or transplantation, and kill them. T cells recognize antigen via a T cell receptor on the surfaces thereof. The T cell receptor recognizes a cell surface molecule MHC (major histocompatibility complex) (HLA (human leucocyte antigen) in human beings), to which a peptide is attached. MHC class I molecules are expressed on all nucleated cells, they preferentially present endogenous cellular peptides. MHC class II molecules are preferentially expressed on professional antigen presenting cells and preferentially present peptides from extracellular antigens. A recognition structure for T-cells can also be called epitope.

The production and display of MHC class I complexes occurs through a peptide processing machinery within cells, whether these are normal, virus infected or transformed to cancer cells. Cells use proteasomes to degrade cytoplasmic proteins into short peptides (1). Some of these peptides are transported from the nucleus or cytoplasm to the endoplasmic reticulum (ER) or to the Golgi apparatus by the transporter associated with antigen processing (TAP) molecule. Once inside the ER or Golgi apparatus, the peptides bind to the MHC class I protein to form a trimolecular complex. This complex is then transported to the cell surface, where it can be recognized by T lymphocyte receptors. Receptors on the surface of a particular type of T lymphocytes, known as $CD8^+$ T lymphocytes, specifically recognize the MHC class I complexes that are formed by the combination of MHC class I proteins and peptides derived from a particular protein, and induce the $CD8^+$ lymphocytes to kill the cells that bear those complexes. If the protein in question is of viral origin, the T-lymphocytes will thus be specific for cells infected with the relevant virus. In a viral infection many of the MHC molecules of the cell are filled with virus peptides instead of peptides from normal cellular proteins.

The peptides are transported into the ER by an intracellular molecular complex called TAP (2). In the absence of a functional TAP-complex, most MHC class I molecules are retained in the ER, and only a small fraction is transported to the cell surface (3-6). This has been studied in cell lines with defects in the TAP genes. The MHC class I molecules of such cells are often referred to as "empty" or "peptide receptive"; they are unstable at physiological temperature but can be stabilized by culture at low temperature or addition of exogenous MHC class I binding peptides (7-9).

TAP is considered crucial for MHC class I restricted CTL responses, because TAP-deficient cells are inefficiently recognized by conventional MHC class I restricted CTL specific for viral-, minor histocompatibility- or tumour antigens (7, 10, 11). In contrast, TAP-deficient cells can be recognized by some allo-MHC class I specific CTL (10, 12, 13). It is unclear whether such allo-specific CTL recognize MHC class

I molecules per se, or MHC class I molecules loaded with TAP-independent peptides. The latter may include peptide species derived from signal sequences (14, 15), or peptides imported to the ER by other TAP-independent mechanisms.

Tumours are composed of cells, which have lost growth control, i.e. they grow without restraint and can invade normal tissue. Tumours can arise in all types of organs. Many research groups make attempts today to induce T cells to recognize and kill tumour cells. The strategy is to find proteins that are unique for the tumour and peptides from these proteins that can attach to different MHC molecules. Such peptides are then used as components in vaccines that should stimulate the immune response to the tumour. One problem is that different tumours contain different proteins, and that MHC molecules and thereby the peptides that attach to the MHC molecules, vary between individuals, as well as between tumours. Another problem is that many tumours have lost parts of the antigen processing mechanism, e.g. TAP, and, therefore, they are not discovered by conventional T cells. They lack antigenicity, and can escape from the immune response (16-19).

TAP-function is thus considered essential for antigenicity, and it has previously been suggested that inhibition of TAP-function in cells should reduce or abrogate T cell responses to the antigens expressed by the cells. This could be induced to prevent autoimmunity. For example, WO 95/15384 describes a TAP inhibitor, a protein ICP47 isolated from Herpes Simplex virus (HSV), and use thereof to inhibit presentation of viral and cellular antigens associated with MHC class I proteins to CD8 T lymphocytes. The present invention is based on a completely opposite concept, namely that prevention of cellular peptide processing leads to increased rather than decreased antigenicity of cells. It has been shown that prevention of TAP-function leads to recognition of novel MHC class I dependent antigens which can be recognized by host T-cells. Induction of such T-cells can prevent cancer growth.

Summary of the invention

The following observations show that substances inducing formation of antigens associated with impaired cellular peptide processing, especially specific MHC class I dependent antigens, such as inhibitors of TAP or of proteasome, can be used as agents for immunization against cancer or as a virus vaccine.

One of the key observations underlying this application is that T-cells could be activated against antigens associated with impaired TAP-function. A TAP-deficient tumour cell from mouse (RMA-S) has previously been produced. This cell line is inefficient in activating responses from cytotoxic T-lymphocytes (example 1, fig 1A and B). After transfection with a stimulatory molecule B7-1 this TAP deficient tumour cell line (RMA-S.B7-1) could activate T cells to a high degree. These T cells recognize a structure independent of TAP, and, therefore, TAP-deficient tumour cells could be killed to a high degree (80% *in vitro*). The inventor has found that TAP-deficient non-transformed normal and tumour cells were capable of inducing a potent CTL response directed against MHC class I dependent epitopes expressed preferentially, if not exclusively, by TAP-deficient cells. B6 mice immunized with irradiated, B7-1 transfected TAP-deficient tumour cells, were protected from outgrowth of a subsequent transplant of TAP-deficient tumour cells, indicating that these novel epitopes may serve as tumour rejection antigens *in vivo*.

A human TAP-deficient tumour cell is also killed by these T cells from mice.

Surprisingly, several TAP-expressing murine tumour cells are also killed by $CD8^+$ cells elicited by TAP-deficient cells (5/5 tested, 4 lymphomas and 1 mastocytoma). The only normal cell type (proliferating T cells, so called Con A blasts) tested from the same mouse are not killed. It was shown that the CTL recognized epitopes independent of TAP-function on tumours that still have TAP-expression, i.e. they can have a relative but not complete impairment of TAP-function.

The inventor demonstrates that the antigens associated with impaired TAP-function can be a shared tumour antigen, which is located on several tumour cell types and hence can be used for tumour immunotherapy.

Detailed description of the invention

One object of the present invention is the use of substances that impair cellular peptide processing, such as inhibitors of TAP or of proteasome for preparation of a pharmaceutical agent or vaccine that can stop or prevent cancer growth or virus infection by stimulating specific T-cells directed against MHC class I dependent antigens associated with impaired cellular processing. Another object of the present invention is the use of substances that impair cellular peptide processing, such as inhibitors of TAP or of proteasome, for preparation of a pharmaceutical agent or vaccine that can stop or prevent cancer growth or virus infection by stimulating immunological effectors, especially $CD8^+$ cells, preferably cytotoxic cells, directed against antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens. The invention also relates to the use of mixtures of such substances.

It can be tested that immunological effector cells are being formed against cancer or virus by culturing cells expressing antigens from cancers or viruses alone in the presence of isolated blood fractions, such as lymphocyte fraction, e.g. as is done in (11). That a substance induces expression of MHC class I dependent antigens associated with impaired cellular peptide expression may be determined by using antibodies against MHC class I. The MHC class I complexes are preferably human complex HLA a, b, or c. Antibodies to measure MHC class I expression can e.g. be obtained from PharMingen (San Diego, California). Measurement is according to standard techniques e.g. a FACS scan analyser.

The invention relates to all means of formation of epitopes/structures or antigens associated with impaired peptide processing, especially antigens associated with im-

paired TAP-function. The invention covers all substances that induce expression of MHC class I dependent antigens associated with impaired cellular peptide processing. The substances according to the invention may therefore be any substance that inhibits the function or the expression of components that take part in the peptide processing of the cell or inhibits an active subfragment of such a substance. More specifically, the invention relates to the use of such substances in eliciting immune responses.

Examples of components that take part in the peptide processing of the cell are e.g. components involved in the translocation of peptides over the ER-membrane such as TAP. Also, substances participating in the cytosolic processing of endogenous proteins such as proteasome are encompassed.

The substance may be a substance that inhibits the function of TAP such as certain viral proteins e.g. TAP-inhibitors e.g. ICP47 of HSV type 1, IE 12 of HSV type 2.

The substance may also be one that inhibits the function of proteasome such as proteasome inhibitors such as the peptide aldehyde Z-Leu-Leu-Leu-H (Peptide Internationals Inc., Louisville, KY).

Moreover, the substance may be a gene encoding an inhibitor of a substance, that takes part in the peptide processing of the cell e.g. an inhibitor of TAP or proteasome.

The substance may also be one that stops the expression of a substance, that takes part in the peptide processing of the cell e.g. TAP or proteasome, such as a nucleotide sequence that is complementary at least in part to the RNA or DNA sequences encoding a substance, that takes part in the peptide processing of the cell. e.g. antisense oligonucleotides or ribozyme destroying RNA.

Anti-sense polynucleotide sequences or analogues thereof can be used to prevent the expression of proteins in vivo or in vitro. If one adds to a cell a large number of strands of a nucleotide sequence that is complementary to the messenger RNA that is transcribed to produce a particular protein, these "anti-sense" strands will hybridize to the mRNA and limit or prevent its translation. This method could be used to limit or prevent the expression of e.g. TAP and/or proteasome. Also, antisense oligonucleotides that hybridize to DNA could be used (20, 21). Thus it is possible to treat cells with antisense TAP (22). Antisense RNA that hybridizes to mRNA can be provided either by adding RNA to cells or introducing gene sequence transcribing antisense RNA (23). Ribozymes that combine enzymatic processes with the specificity of antisense base pairing may also be used (24). These techniques are discussed in (25).

TAP inhibitors may be produced according to WO 95/15384.

One purpose of the invention is to stimulate cells in a patient suffering from cancer or certain viruses to express antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens. The virus may be one that impairs peptide processing, e.g. the TAP-function such as Herpes Simplex. This could be done in vitro or in vivo.

When done in vivo the patient is given substances that impair cellular peptide processing. Thus, compositions containing such a substance e.g. inhibitors of TAP or proteasome may be given. The patient can be vaccinated with e.g. ribozyme, antisense RNA, antisense DNA and/or antisense oligonucleotides against the expression of a substance that takes part in cellular peptide processing or a gene encoding an inhibitor of such a substance i.e. a gene encoding a substance that impairs cellular peptide processing.

DNA can be introduced directly in the cells of a living host by so called DNA immunization. This involves many different techniques e.g. intramuscular injection,

intradermal injection (particle bombardment where cells in the epidermis are transfected with DNA-coated gold beads) or delivered by various vectors such as recombinant Shigella. Many other techniques are developed in different laboratories. Also RNA and oligonucleotides may be given in this way (26, 27).

Another object of the invention are cells, that have been treated to express antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens, to be used for preparing a pharmaceutical or vaccine against cancer or virus infections.

These cells may be non-mammalian cells with impaired peptide processing e.g. cells that lack TAP and/or proteasome function and to which human MHC class I molecules have been transfected e.g. insect cells (28).

The invention preferably relates to mammalian cells and especially to autologous mammalian cells that have been treated to express MHC-class I dependent antigens associated with impaired cellular peptide processing.

The cells may be chosen from hematopoetic or dendritic cells. Autologous cells are especially preferred.

When cancers are to be treated the cells can also be autologous cells, especially healthy cells from the affected tissue/organ.

These cells, that express MHC class I dependent antigens associated with impaired peptide processing, will then be injected into a patient in order to stimulate T cells to react on these antigens

Another object of the invention is a process for induction of antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens in mammalian cells, characterized in that:

a) the cells are treated with agents that inhibit substances that take place in the cellular peptide processing in mammalian cells e.g. TAP-inhibiting or proteasome inhibiting agents or

b) a sequence that codes for such an inhibiting agent is introduced into the DNA of the cell or

c) a nucleotide sequence that is complementary at least in part to the mRNA or DNA sequences encoding a substance that takes place in the cellular peptide processing in mammalian cells e.g. TAP or proteasome is introduced into the DNA of the cell or

d) the cells are treated with an appropriate ribozyme and

e) when non-mammalian cells are used, transfection thereof with human MHC class I molecules and

f) the cells may be irradiated with an appropriate dose with e.g. γ-irradiation from e.g. Cs^{137}.

In order to make target cells to express a foreign gene product the DNA has to be incorporated in the target cells. Plasmid DNA can be incorporated in the target cells by transfection, e.g. by electroporation, lipofection, calcium precipitation or particle resolution. Vehicles may be needed such as liposomes e.g. DOTAP (Boehringer Manheim).

Another possibility to incorporate the foreign DNA in the target cells is the use of retroviruses, where the DNA is enveloped in a protein. In the case of retroviruses the DNA will be stably incorporated in the genome with proliferating cells. (29)

Mammalian cells may be cultured in a medium suitable for eucaryotic cells e.g. RPMI 1640 containing bovine serum albumin.

Dendritic cells can be sorted from the peripheral blood by e.g. immunomagnetic sorting to molecules such as CD34 or CD14. Magnetic beads can be obtained from Dynal. They are grown in vitro in suitable medium, e.g. IMDM (Life Technologies, Inc., Grand Island, NY) with appropriate supplements (30) and various adjuvants to

improve development and immunogenicity. Examples of adjuvants are cytokines such as Granulocyte-Macrophage colony stimulating factor (GM-CSF), IL-4, Tumour Necrosis Factor alfa (TNF-alfa), stem cell factor (SCF) or Transforming Growth Factor - beta (TGF-beta), antibodies to MHC Class II or CD40 (which enhance B7 expression) or genes for costimulatory molecules.

Cells, that have been treated to express antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens, e.g. with TAP or proteasome inhibitors may be used for activation in vivo or in vitro of T cells against MHC class I dependent antigens associated with impaired cellular processing. The in vivo procedure is described above. The in vitro procedure could be e.g. as follows:

a) cells are treated to express MHC-class I dependent antigens associated with impaired cellular peptide processing as described above
b) T cells are isolated and stimulated in vitro with the cells obtained in step a and
c) activated T cells are given to the patient.

Stimulation of T-cells in vitro with dendritic cells is done according to current standard procedures, e.g. T-cells are sorted out from peripheral blood and cultured in the presence of dendritic cells in appropriate media and appropriate additives e.g. MEM media and IL-2 (30, 31).

According to one aspect of the invention lymphoid cells such as T-cells e.g. TCL, preferably $CD8^+$ T lymphocytes, activated against antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens, are used for preparing a pharmaceutical or vaccine against cancer or virus infections.

Optimal conditions for human T cells and dendritic cells are e.g. as in (30, 31). T cell activation can be enhanced by treating the cells with cytokines, e.g. GM-CSF or antibodies to e.g. CD40 or MHC class II, which enhance B7 expression. Cytokines and antibodies can be obtained from ImmunoKontakt, Switzerland.

These cells can be tested, e.g. with T-cell clones directed against TAP-inhibited cells. Inhibition of TAP-function can be measured by peptide transporter assays, e.g. as in (32).

The invention also relates to a kit, for use in a process for induction of antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens in cells, characterized in that it comprises an active dose of a substance that stimulates formation of MHC peptide complex such as an inhibitor of TAP or of proteasome or a nucleotide sequence that is at least in part complementary to the mRNA or DNA sequences encoding proteasome or TAP.

The kit may further comprise cytokines and genes for costimulatory molecules.

The invention also concerns a pharmaceutical composition or a vaccine comprising a pharmaceutically effective dose of a substance that induces antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens such as an inhibitor of TAP or of proteasome or a gene encoding a proteasome or a TAP inhibitor or a nucleotide sequence that is complementary at least in part to the mRNA or DNA sequences encoding proteasome or TAP e.g. antisense nucleotides or ribozyme together with a pharmaceutically acceptable adjuvant.

Pharmaceutical compositions of the present invention contain a physiologically acceptable carrier together with at least one substance that impairs cellular peptide processing as described herein, dissolved or dispersed therein as an active ingredient.

As used herein, the term "pharmaceutically acceptable" represents that the materials are capable of administration to or upon a human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as sterile injectables either as liquid solutions or suspensions, aqueous or non-aqueous, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The pharmaceutical composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic; inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylaminoethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, propylene glycol, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerine, vegetable oils such as cottonseed oil, organic esters such as ethyl oleate, and water-oil emulsions.

All technical and scientific terms used herein are, unless otherwise defined, intended to have the same meaning as commonly understood by one of ordinary skill in the art. Techniques employed herein are those that are known to one of ordinary skill in the art unless stated otherwise. Publications mentioned herein are incorporated by reference.

The following observations show that substances inducing formation of specific MHC class I dependent antigens, such as inhibitors of TAP or of proteasome, can be used as agents for immunization against cancer or as a virus vaccine.

Summary of the observations:

- $CD8^+$ T-cells can be activated against epitopes recognized preferentially, if not exclusively, expressed by TAP-deficient cells. This is shown by immunizing B6 mice with syngenic TAP-deficient tumour transformed cells (TAP-deficient RMA-S tumour cells transfected with the costimulatory molecule B7-1. The cells are designated RMA-S.B7-1) and non-transformed spleen cells from TAP1 -/- mice (both alleles of the TAP gene knocked out) and testing the elicited CTL on different TAP-deficient and TAP-expressing target cells (Examples 1-3).
- The recognition of epitopes is dependent of MHC class I expression. This is shown by testing elicited CTL on cells expressing different combinations of MHC class I defects (Example 2).
- The epitopes are recognized on TAP-deficient murine tumour transformed and non-transformed cells as well as on a TAP-deficient human tumour transformed cell line (Examples 2-3).
- Surprisingly, syngenic TAP-deficient cells, both RMA-S.B7-1 tumour cells as well as spleen cells and dendritic cells from TAP1 -/- mice, elicit T cells that recognize

several different syngenic TAP-expressing murine tumour cells while syngenic nontumour transformed cells are not killed (Examples 6-8).

- The epitopes recognized on TAP-expressing tumour cells are epitopes independent of TAP-function (Example 5).
- Immunization with syngenic TAP-deficient cells can protect against tumour growth in vivo of both TAP-deficient and TAP-expressing tumour cells (Examples 4, 9).

In conclusion, the inventor demonstrates that the antigens associated with impaired TAP-function can be a shared tumour antigen, which is located on several tumour cell types and hence can be used for tumour immunotherapy.

In the TAP1-/- mice used in the experiments the TAP1 gene has been deleted by genetic manipulation, i.e. complete TAP-inhibition. From the data presented it is easy to predict that CTL will be elicited in a human host with autologous or MHC matched cells treated with various TAP-inhibitors. According to the data presented these CTL may be used in immunotherapy of both TAP-deficient and TAP-expressing tumours. From the literature it is known that several viruses can inhibit antigen presentation and hence escape conventional CTL recognition. For example, Herpes Simplex inhibits TAP-function (33, 34). Therefore CTL elicited by TAP-inhibited cells may also be used as a therapeutical agent in viral infections where TAP-function is inhibited as above. The presentation of MHC class I molecules and TAP-dependent peptides at the cell surface is a complex process depending on many factors, of which translocation of peptides over the ER-membrane by TAP-molecules is one. Another crucial step is cytosolic processing of endogenous proteins by the proteasome. Cells that lack components of the proteasome have a similar phenotype to TAP-deficient cells, i.e. deficient generation of antigenic peptide. Proteasome inhibitors block MHC class I restricted antigen presentation and assembly of MHC class I molecules, the assembly can be reconstituted by exogenously added peptides (1, 35-37). It is likely that the epitopes associated with impaired TAP-function will be exposed also upon inhibition of the proteasome and could with a more general term be named MHC class I dependent epitopes associated with im-

paired peptide processing. Therefore the invention also relates to the elicitation of T cells with proteasome inhibitors. It is also easy to envisage that other yet undefined factors are necessary for formation of the MHC/TAP-dependent-peptide complex and that inhibition of those factors will result in a similar phenotype exposing epitopes associated with impaired TAP-function.

This underlying discovery reveals the existence of MHC class I restricted CTL epitopes that are recognized preferentially, if not exclusively, in the absence of a functional TAP complex. B6 mice immunized with B7-1 transfected TAP-deficient RMA-S tumour cells or spleen cells from TAP1 -/- mice, generated a potent CTL response against both RMA-S tumour cells and TAP1 -/- Con A blasts targets. In contrast, TAP-expressing RMA-S.TAP2 tumour cells and B6 Con A blasts were largely resistant to lysis by these CTL. RMA-S.B7-1 immunized mice were protected from outgrowth of RMA-S tumour transplants, indicating that these epitopes associated with impaired TAP-function can serve as tumour rejection antigens in vivo. Surprisingly, several TAP-expressing tumours were also sensitive to the elicited CTL while non-tumour transformed cells were resistant It was shown that the CTL recognized epitopes independent of TAP-function on tumours that still have TAP-expression, i.e. they can have a relative but not complete impairment of TAP-function. The difference in sensitivity between RMA and RMA-S.TAP2 which are both TAP-expressing is probably due to that RMA-S.TAP2 is a transfectant with TAP and hence can overexpress TAP compared to the levels in the original tumour RMA.

The TAP1 -/- mice used in the experiments described have been backcrossed at least 6 times. Hence immunization of B6 mice with the TAP1 -/- cells can be regarded as a syngenic immunization with completely TAP-inhibited cells. From the present data it is easy to predict that it will possible to elicit CTL in a human host with autologous TAP-inhibited cells. According to the data presented these CTL may be used in immunotherapy of both TAP-deficient and TAP-expressing tumours. These CTL may also be used as therapeutical agent in viral infections where TAP-function is

inhibited (33, 34). This study is done with cells of lymphoid origin. Preliminary data suggests that also a non-lymphoid cell line, the H-2Kb transfected mastocytoma P815 (data not shown) is killed by RMA-S.B7-1 elicited CTL. It is under investigation whether tumours of non-hematopoetic origin can be recognized by CTL elicited by TAP-inhibited hematopoetic cells, or whether TAP-inhibited cells from the tissue of origin for the tumour, has to be used for immunization.

The substances according to the invention may be used against tumours, preferentially that have lost expression of TAP, but also towards TAP-expressing tumours. The substances may also be used against certain virus infections where TAP-function is inhibited by viral proteins, e.g. in Herpes Simplex virus infected cells.

Brief description of the drawings:

Figure legends

Figure 1. Immunization of B6 mice with RMA-S.B7-1 cells elicits $CD8^+$ CTL that recognize non-transfected RMA-S cells. (A and B) B6 mice were immunized in vivo and splenocytes were restimulated in vitro with RMA-S tumour cells (O) or RMA-S cells transfected with B7-1 (◆) and tested for cytotoxicity against RMA-S target cells. Two experiments are shown, panel A is representative of 4/6 experiments, panel B is representative of the remaining experiments. (C) CTL generated as above were depleted with anti-CD8 antibodies and complement (▲) or anti-CD4 antibodies and complement (D) and tested for cytotoxicity against RMA-S target cells. One representative experiment out of three is shown.

Figure 2. Recognition of epitopes by RMA-S.B7-1 elicited CTL requires the absence of TAP-function and the presence of MHC class I molecules in the target cell. B6 mice were immunized in vivo and splenocytes were restimulated in vitro with RMA-S.B7-1 cells and tested for cytotoxicity against (A) RMA-S (O) and RMA-S.TAP-2 (●), (B) Con A blasts from B6 (□), TAP1 -/- (■), β_2m -/- (D), and

TAP1/β_2m -/- mice (▲) and (C) T2 (▽) and T2K^b (▼) cells. One representative experiment out of three is shown.

Figure 3. Immunization with TAP1 -/- splenocytes elicits CTL that recognize TAP1 -/- Con A blasts and RMA-S tumour cells. B6 mice were immunized in vivo and splenocytes were restimulated in vitro with splenocytes from TAP1 -/- mice and tested for cytotoxicity against RMA-S (○), B6 Con A blasts (□) and TAP1 -/- Con A blasts (■). One representative experiment out of four is shown.

Figure 4. Immunization of B6 mice with RMA-S.B7-1 cells protects from outgrowth of RMA-S tumour cells. B6 mice were given 10^6 live RMA-S tumour cells after immunization with PBS (◇), RMA-S (○) or RMA-S.B7-1 (◆). The figure represents the accumulated data of four separate experiments (three for RMA-S.B7-1) with 4-6 mice/immunization group in each experiment.

Figure 5. The TAP-expressing tumour RMA expresses epitopes independent of TAP-function. B6 mice were immunized in vivo and splenocytes were restimulated in vitro with RMA-S.B7-1 tumour cells and tested for cytotoxicity against (A) RMA-S (□), RMA (◇), RMA-S.TAP2 (○) or B6 Con A blasts (D), (B) RMA to which cold B6 Con A blasts (□) or TAP1-/- Con A blasts(◇) in designated ratios were added.

Figure 6. Several TAP-expressing tumour cell lines are killed by CTL elicited by RMA-S.B7-1, while non-tumour transformed TAP-expressing cells are resistant. B6 mice were immunized in vivo and splenocytes were restimulated in vitro with RMA-S.B7-1 tumour cells and tested for cytotoxicity against RMA-S (□), EL-4 (◇), ALC (○), C4425- (D), P815 (▽), B6 Con A blasts (■) and TAP1-/- Con A blasts (◆). One representative experiment out of three is shown.

Figure 7. Immunization with TAP1 -/- dendritic cells elicits CTL that recognize several TAP-expressing tumour transformed cells. B6 mice were immunized in vivo

with dendritic cells from TAP1 -/- mice and splenocytes were restimulated in vitro with splenocytes from TAP1 -/- mice and tested for cytotoxicity against RMA-S (□), EL-4 (◇), ALC (O), RMA (D), P815K^b (▲), 26E1Nmyc (▼) and B6 Con A blasts (■). One experiment of two is shown.

Figure 8. Immunization of B6 mice with RMA-S.B7-1 cells protects from outgrowth of several TAP-expressing tumour cells. B6 mice were immunized with PBS (◇) or RMA-S.B7-1 (□), or CD8-/- mice were immunized with PBS (D) or RMA-S.B7-1 cells (O) and given in (A) 10^5 RMA tumour cells, (B) 10^2 EL-4 tumour cells (C) 10^3 ALC tumour cells. The figure represents one experiments with 4-6 mice/immunization group.

The invention will now be described by reference to some non limiting examples

Materials and Methods

Mice. All mice were bred and maintained at the Microbiology and Tumour Biology Center, Karolinska Institute. The generation and characterization of TAP1 -/-, β_2-microglobulin (β_2m) -/- and TAP1/β_2m -/- mice has been described (38-40). The TAP1 -/- and β_2m -/- mice used in the present study have been backcrossed to B6 (C57BL/6) at least six times. Animal care was in accordance with institutional guidelines.

Cell lines. T2K^b is a H-2K^b (mouse MHC allele (type)) transfected subline of the TAP1/2 deficient mutant human cell line T2 (41). All cell lines were grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with penicillin-streptomycin and 5% FCS (Fetal Calf serum) at 37°C and 5% CO_2.

Antibodies. B7-1 (RMA S.B7-1) expression was assessed either with the CTLA-4Ig fusion protein (42), a kind gift from Dr P. Lane, Basel Institute for Immunology.

Basel, Switzerland, and a FITC (Fluoroscein isothiocyanate)-conjugated anti-human IgG antibody (Dako, Glostrup, Denmark), or with a biotinylated anti-B7-1 monoclonal antibody 16-10A1 (PharMingen, San Diego, CA) and NEUTRALITE avidin-FITC (Southern Biotechnology Associates Inc., Birmingham, AL).

Cold target competition assay. For the cold target competition assay cold (unlabeled) Con A blasts were incubated at different concentrations with a constant number of effector cells and 5 x 10^3 ^{51}Cr-labeled target cells.

Immunization with bone marrow derived dendritic cells. Bone marrow derived derived dendritic cells were obtained from TAP1-/- mice using the protocol of Inaba and colleagues (43) with the following alterations. Bone marrow cells were cultured in Dulbecco's modified Eagles Medium containing 10 % supernatant from the GM-CSF (Granulocyte Macrophage Colony Stimulating Factor secreting cell line X63 (a kind gift from Dr C.Watts, Univ Dundee, Dundee, UK) and 20 % FCS. The culture media was replaced every third day, and the cells were replated on day 7. On the eight day, the non-adherent cells were used for in vivo immunization. 10^5 cells were given intraperitoneally, splenocytes were restimulated 10 days after in vivo immunization.

Examples

A. CD8$^+$ T cells can be elicited to MHC class I restricted epitopes associated with impaired TAP-function. Immunization with a TAP-deficient cell can protect against tumour growth in vivo.

Example 1

RMA-S cells transfected with B7-1 (CD80) elicit CTL that recognize non-transfected RMA-S cells.

B6 mice were immunized with three weekly s.c. injections of 10^7 irradiated (100 Gray (Gy)) tumour cells. Tumours used were serially passaged as ascites cell lines in 4 Gy irradiated mice. The tumour cells were TAP-2 deficient ones, called RMA-S cells (derived from the Rauscher leukaemia virus-induced mouse T-cell lymphoma RBL-5 of B6 origin (44)), which were transfected with B7-1, i.e. 2 x 10^6 RMA-S cells were incubated with 10 µl LIPOFECTAMINE (Life Technologies, Gaithersburg, MD) and 1 µg of the murine B7-1 gene cloned in a pSRIneo plasmid (45), a gift from Bristol Meyers Inc, Seattle, to Prof Klas Kärre. Transfected cells were selected on GENETICIN (Life Technologies, Gaithersburg, MD) at a concentration of 1 mg/ml. The 1 % most positive fraction of the B7-1 expressing RMA-S cells were sorted on a FACS VANTAGE cell sorter (Becton Dickinson, Mountain View, CA) and designated RMA-S.B7-1.

This transfection resulted in strong and reproducible lysis of non-transfected RMA-S targets (Fig. 1 A and B). The lysis could be abrogated by pre-treatment of effectors with anti-CD8 antibodies (CD8 is a marker of the cell surface of CTL) and complement (Fig. 1 C). The complement-mediated depletion of effector populations was conducted as follows: 20 x 10^6 effector cells were incubated in 200 µg of anti-CD8 antibody (a mixture of 169.4 and 156.7.7 in 1 ml PBS, or 200 µg of anti-CD4 antibody (YTS191) in 1 ml PBS for 60 min at 4°C. All antibodies were generously provided by Dr H. Waldman, University of Cambridge, Cambridge, UK. The cells were washed once and incubated with rabbit complement (Pel-Freeze Biologicals, Brown Deer, WI) diluted 1:8, for 75 min in 37°C.

This demonstrates that it is possible to generate CTL against syngenic TAP-deficient cells. Control cell lines, YAC-1 (a Moloney virus induced T cell lymphoma of A/Sn background) and P815 (a Methylcholantrene induced mastocytoma of DBA/2 background), as well as Concanavalin A (Con A) blasts from BALB/c mice were resistant to lysis by these CTL (data not shown). The generation of Con A-activated T-cell blasts was as follows: Spleen cells were incubated for 48 h at 2 x 10^6 cells/ml in

α-MEM medium (Life Technologies, Gaithersburg, MD) supplemented with penicillin-streptomycin, 10 % FCS, 10 mM HEPES (Life Technologies, Gaithersburg, MD), 3 x 10^{-5} M 2-ME (2-Mercaptoethanol) (Sigma, St. Louis, MO) and 3 μg/ml of Con A (Sigma, St. Louis, MO). Before use as targets in a standard 4 h ^{51}Cr cytotoxicity assay, dead cells were removed by centrifugation on a LYMPHOPREP gradient (Nycomed, Oslo, Norway).

TAP-2 deficient RMA-S tumour cells were inefficient in eliciting cytotoxic responses in B6 mice (Fig. 1 A and B).

Example 2

CTL recognition requires the presence of MHC class I molecules and the absence of TAP in the target cell.

A TAP-2 transfectant of RMA-S (RMA-S.TAP2 cells, also referred to as RMA-S II 5.9 cells, were derived by transfection of RMA-S with the murine TAP-2 gene (46)) was virtually resistant to lysis by RMA-S.B7-1 elicited CTL (Fig. 2 A) as in Example 1. This indicated that the epitopes were recognized preferentially in cells devoid of TAP expression. In line with this, Con A blasts from TAP1 -/- mice were highly sensitive to lysis, whereas Con A blasts from B6 mice were resistant to lysis. Con A blasts from TAP1/β_2m -/- (double mutant) mice were resistant to lysis, suggesting an MHC class I dependence in the CTL recognition of epitopes (Fig. 2 B).

Indeed, the human TAP-deficient cell line T2 transfected with H-2K^b was sensitive to lysis by RMA-S.B7-1 elicited CTL, while non-transfected T2 cells were resistant (Fig. 2 C). Taken together, these results indicate that at least part of the response elicited by RMA-S.B7-1 is MHC class I specific or restricted and directed against epitopes expressed preferentially, if not exclusively, by TAP-deficient cells. At least some of the epitopes could be recognized on both non-transformed and transformed lymphoid cells, and on cells of both human and murine origin. These epitopes will be referred to as epitopes associated with impaired TAP-function.

Example 3

TAP1 -/- splenocytes elicit CTL that recognize TAP1 - - Con A blasts and RMA-S tumour cells.

As shown above, CTL elicited by RMA-S.B7-1 killed TAP1 -/- Con A blasts. Accordingly, immunization of B6 mice with splenocytes from TAP1 -/- mice yielded cytotoxic cells that efficiently lysed TAP1 -/- Con A blasts and RMA-S tumour cells while B6 Con A blasts and RMA-S.TAP2 were killed at markedly reduced levels (Fig. 3; data not shown). The killing of the TAP-deficient cells was also seen with effectors from mice depleted of NK (natural killer) cells (data not shown). B6 mice were immunized with two weekly s.c. injections of $50x10^6$ irradiated (20 Gy) spleen cells.

Example 4

Generation of primary CTL by the epitopes associated with impaired TAP-function.

Stimulation in vitro, without prior in vivo immunization, of B6 spleen cells with RMA-S.B7-1 and TAP1 -/- spleen cells was conducted as follows: Single cell suspensions of spleens from immunized or non-immunized mice were prepared. 20 x 10^6 effector cells were incubated with 2 x 10^6 irradiated tumour cells or 20 x 10^6 irradiated spleen cells. Cultures were kept in 10 ml of RPMI 1640 medium supplemented with penicillin-streptomycin, 10 % FCS, 3 x 10^{-5} M 2-ME, 1 mM sodium pyruvate, 0,1 mM non-essential amino acids and 2 mM L-glutamine at 37° C and 5 % CO_2 for five days.

This stimulation reproducibly resulted in cytotoxic responses against RMA-S and TAP1 -/- Con A blasts targets, while RMA-S.TAP2 and B6 Con A blasts were considerably less sensitive. The in vitro cytotoxicity assay was conducted as follows:

Target cells were labelled with ^{51}Cr and resuspended in cell culture medium. 5 x 10^3 target cells were added to each well followed by addition of effector cells. The cells were incubated for 4 h at 37°C and supernatants were harvested. Radioactivity was measured in a Pharmacia-LKB γ-counter, and specific lysis was calculated [(CPM (counts per minute) released with effector cells-CPM released without effector cells) / (CPM released by detergent-CPM released without effector cells)] x 100. Results with more than 20 % spontaneous lysis were discarded.

Stimulation in vitro with non-transfected RMA-S cells yielded in some experiments lower levels of lysis (Table I; data not shown). The killing of RMA-S and TAP1 -/- Con A blast targets was abrogated by pre-treatment of effectors with anti-CD8 antibody and complement (data not shown).

Example 5

Immunization of B6 mice with RMA-S.B7-1 protects against RMA-S tumour growth.

To address whether it was possible to elicit a protective immune response against a TAP-deficient tumour cell line, we immunized B6 mice with RMA-S cells, RMA-S.B7-1 cells or PBS (phosphate buffered saline). After three weekly immunizations, mice were challenged with 10^6 live RMA-S tumour cells s.c., a dose previously found to overcome the NK mediated rejection of RMA-S (41).. The in vivo tumour growth was as follows: B6 mice were immunized as described. One week after the last immunization, mice were given 10^6 live tumour cells s.c. and growth was followed weekly by palpation. For each mouse, the experiment was terminated when the tumour reached a diameter of 20 mm.

Eighty nine percent of the mice (17/19 mice) immunized with PBS developed progressively growing tumours within three weeks after challenge. In contrast, only eight percent (1/13) of the mice immunized with RMA-S.B7-1 developed tumours.

Mice immunized with RMA-S were partially protected: Fifty five percent of the mice (10/18 mice) developed progressively growing tumours (Fig. 4).
The RMA-S.B7-1 mediated protection from tumour growth was also seen in NK 1.1. depleted mice (data not shown).

B. Epitopes associated with impaired TAP-function is a shared tumour antigen found on several TAP-expressing tumours but not on non-tumour transformed cells. Immunization with a TAP-deficient cell protects in vivo against tumour growth of TAP-expressing tumour cells.

Example 6

The TAP-expressing tumour cell RMA is recognized by CTL directed to epitopes associated with impaired TAP-function.

Surprisingly, CTL elicited by RMA-S.B7-1 reproducibly killed the TAP-expressing parental tumour cell of RMA-S, RMA. The lysis levels were markedly higher than with the TAP-transfected tumour cell line RMA-S.TAP2, but still below the levels of lysis of the TAP-deficient cell RMA-S (Fig 5A). As described above, no killing was observed of TAP-expressing B6 Con A blasts. In cold target inhibition experiments with Con A blasts from B6 and TAP-/- mice, the latter were more efficient in inhibiting lysis of RMA by CTL elicited by RMA-S.B7-1 (Fig 5B), demonstrating that at least part of the killing of RMA by these CTL was dependent on recognition of epitopes independent of TAP-function. Control experiments where hot and cold targets were incubated without effectors did not result in any lysis by either cold target (data not shown).

Example 7

Several TAP-expressing tumour transformed cell lines are killed by CTL elicited by RMA-S.B7-1 while non-transformed TAP-expressing cells are resistant.

A possible explanation to the observed killing of RMA is that some tumour cells may have a relative deficiency in TAP-function, i.e. they express suboptimal levels of TAP-proteins. To investigate whether epitopes associated with impaired TAP-function could be a shared antigen we tested other H-2^b expressing tumour cell lines transformed by different agents, for killing by RMA-S.B7-1 elicited CTL. Indeed, all tumour cells tested, the carcinogen induced (9,10-dimethyl-1,2-benzanthracene) EL-4 (American Type Culture Condition, Rockville, MD), the Radiation Leukaemia virus induced ALC (generously provided by Dr Wen Tao, Karolinska Institute, Sweden) and 26E-1Nmyc tumour cells (a gift from Dr Santiago Silva, Karolinska Institute, Sweden) were killed by RMA-S.B7-1 elicited CTL. 26E1Nmyc is a spontaneous lymphoma from mice transgenic for EBNA-1 and N-myc (derived by crossing EBNA-1 and N-myc transgenic mice (47, 48)). A β_2m-negative variant of EL-4, as well as P815 tumour cells (a mastocytoma of a H-2^d origine obtained from American Type Culture Condition, Rockville, MD) were not killed by RMA-S.B7-1 elicited CTL (Fig 6). Of the cells described above, only H-2^d positive P815 and 26E-1Nmyc cells were killed by B6 CTL elicited by Balb/c splenocytes (data not shown) showing that the tumour cells were not generally sensitive for all CTL tested.

Example 8

TAP-deficient nontransformed cells elicit CTL that recognize several TAP-expressing tumour cell lines but not non-transformed cells.

CTL from B6 mice immunized with splenocytes or cultured dendritic cells from TAP1 -/- mice also killed RMA, ALC, 26E-1Nmyc, EL-4 (to lower levels), P815 cells transfected with H-$2K^b$ and TAP1 -/- Con A blasts. B6 Con A blasts were not killed by these CTL (Fig 7). This strengthens the notion that TAP-expressing tumour transformed but not non-transformed cells express epitopes associated with TAP-deficiency. The lysis levels were lower than those observed with RMA-S.B7-1 which is probably due to the high levels of B7 expression on RMA-S.B7-1.

Example 9

Immunization with RMA-S.B7-1 protects from tumour growth of several TAP-expressing tumours.

B6 mice were immunized with RMA-S.B7-1 or non-immunized. One week after the last immunization mice were given 10^5 RMA tumour cells or 10^2 EL-4 tumour cells or 10^3 ALC tumour cells. In the immunized groups only 20 % of mice developed tumours while all mice in the non-immunized groups developed progressively growing tumours. For RMA and EL-4 the protection observed in the immunized mice was not seen in mice deficient of CD8, demonstrating that this protection was mediated by $CD8^+$ CTL (Fig 8 A-C).

References

1. Groettrup, M., A. Soza, U. Kuckelkorn and P.-M. Kloetzel. 1996. Peptide antigen production by the proteasome: complexity provides efficiency. *Immunol. Today.* 9:429-435.

2. Heemels, M.T, and H. Ploegh. 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. *Annu. Rev. Biochem.* 64:463-491.

3. Powis, S.J., A.R. Townsend, E.V. Deverson, J. Bastin, G.W. Butcher, and J.C. Howard. 1991. Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature.* 354:528-531.

4. Spies, T, and R. DeMars. 1991. Restored expression of major histocompatibility class I molecules by gene transfer of a putative peptide transporter. *Nature.* 351:323-324.

5. Degen, E., M.F. Cohen-Doyle, and D.B. Williams. 1992. Efficient dissociation of the p88 chaperone from major histocompatibility complex class I molecules requires both β_2-microglobulin and peptide. *J. Exp. Med.* 175:1653-1661.

6. Suh, W.K., E.K. Mitchell, Y. Yang, P.A. Peterson, G.L. Waneck, and D.B. Williams. 1996. MHC class I molecules form ternary complexes with calnexin and tap and undergo peptide-regulated interaction with TAP via their extracellular domains. *J. Exp. Med.* 184:337-348.

7. Townsend, A., C. Öhlén, J. Bastin, H.G. Ljunggren, L. Foster, and K. Kärre. 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature.* 340:443-448.

8. Ljunggren, H.G., N.J. Stam, C. Öhlén, J.J. Neefjes, P. Höglund, M.T. Heemels, J. Bastin, T.N. Schumacher, A. Townsend, and K. Kärre. 1990. Empty MHC class I molecules come out in the cold. *Nature.* 346:476-480.

9. Day, P.M., F. Esquivel, J. Lukszo, J.R. Bennink, and J.W. Yewdell. 1995. Effect of TAP on the generation and intracellular trafficking of peptide-receptive major histocompatibility complex class I molecules. *Immunity.* 2:137-147.

10. Öhlén, C., J. Bastin, H.G. Ljunggren, L. Foster, E. Wolpert, G. Klein, A.R. Townsend, and K. Kärre. 1990. Resistance to H-2-restricted but not to allo-H2-specific graft and cytotoxic T lymphocyte responses in lymphoma mutant. *J. Immunol.* 145:52-58.

11. Franksson, L., M. Petersson, R. Kiessling, and K. Kärre. 1993. Immunization against tumour and minor histocompatibility antigens by eluted cellular peptides loaded on antigen processing defective cells. *Eur. J. Immunol.* 23:2606-2613.

12. Aosai, F., C. Öhlén, H.G. Ljunggren, P. Höglund, L. Franksson, H. Ploegh, A. Townsend, K. Kärre, and H.J. Stauss. 1991. Different types of allospecific CTL clones identified by their ability to recognize peptide loading-defective target cells. *Eur. J. Immunol.* 21:2767-2774.

13. Rötzschke, O., K. Falk, S. Faath, and H.G. Rammensee. 1991. On the nature of peptides involved in T cell alloreactivity. *J. Exp. Med.* 174:1059-1071.

14. Wei, M.L, and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature.* 356:443-446.

15. Henderson, R.A., A.L. Cox, K. Sakaguchi, E. Appella, J. Shabanowitz, D.F. Hunt, and V.H. Engelhard. 1993. Direct identification of an endogenous peptide

recognized by multiple HLA-A2.1-specific cytotoxic T cells. *Proc. Natl. Acad. Sci.* U. S. A. 90:10275-10279.

16. Restifo, N.P., F. Esquivel, Y. Kawakami, J.W. Yewdell, J.J. Mule, S.A. Rosenberg, and J.R. Bennink. 1993. Identification of human cancers deficient in antigen processing. *J. Exp. Med.* 177:265-272.

17. Cromme, F.V., J. Airey, M.T. Heemels, H.L. Ploegh, P.J. Keating, P.L. Stern, C.J. Meijer and J.M. Walboomers. 1994. Loss of transporter protein, encoded by the TAP-1 gene, is highly correlated with loss of HLA expression in cervical carcinomas. *J. Exp. Med.* 179:335-340.

18. Seliger, B., A. Hohne, A. Knuth, H. Bernhard, T. Meyer, R. Tampe, F. Momburg, and C. Huber. 1996. Analysis of the major histocompatibility complex class I antigen presentation machinery in normal and malignant renal cells: evidence for deficiencies associated with transformation and progression. *Cancer Res.* 56:1756-1760.

19. Garrido, F., T. Cabrera, A. Concha, S. Glew, F. Ruiz-Cabello, and P.L. Stern. 1993. Natural history of HLA expression during tumour development. *Immunol. Today.* 14:491-499.

20. Crooke, S.T., Bennett, C.F. 1996. Progress in antisense oligonucleotide therapeutics. *Annual Review of Pharmacology & Toxicology* 36:107-129.

21. Zon, G. 1995. Antisense phosphorothioate oligodeoxinucleotides: introductory concepts and possible mechanisms of toxicity. *Toxicology Letters*, Dec, 82-83:419-424.

22. Nair, S.K., D. Snyder, and E. Gilboa. 1996. Cells treated with TAP-2 antisense oligonucleotides are potent antigen-presenting cells in vitro and in vivo. *J. Immunol.* 156:1772-1780.

23. Sharma H.W., Narayanan, R. 1996. The NF-kappaB transcription factor in oncogenesis. *Anticancer Research*, Mar-Apr, 16(2):589-596.

24. Ohkawa, J., Koguma, T., Kohda, T., Taira, K. 1995. Ribozymes: from mechanistic studies to applications in vivo. *Journal of Biochemistry*, Aug, 118(2):251-258.

25. Putnam, D.A. 1996. Antisense strategies and therapeutic applications. *American Journal of Health-System Pharmacy*, Jan, 15;53(2):151-160.

26. Ulmer J.B., J.C. Sadoff and M.A. Liu. 1996. DNA vaccines. *Current Opinion in Immunology*. 8:531-536.

27. Tascon, RE., Colston MJ., Ragno, S., Stavropoulus, E., Gregory, D., Lowrie, DB. 1996. Vaccination against tuberculosis by DNA injection. *Nature Medicine*, Aug, 2(8):888-892.

28. Uebel S., T.H. Meyer, W Kraas, S. Kienle, G. Jung, K.H. Wiesmuller, and R. Tampe. 1995. Requirements for peptide binding to the human transporter associated with antigen processing revealed by peptide scans and complex libraries. *J. Biological Chemistry* 270 (31):18512-18516.

29. WO 96/33272.

30. Strobl H., E. Riedl, C. Scheinecker, C. Bello-Fernandez, W.F. Pickl, K. Rappersberger, O. Majdic and W. Knapp. 1996. TGF-beta1 promotes in vitro development of dendritic cells from $CD34^+$ hemopoetic progenitors. *J. Immunology*. 157(4):1499-507.

31. Kiertscher S.M., and Roth M.D. 1996. Human $CD14^+$ leukocytes acquire the phenotype and function of antigen-presenting dendritic cells when cultured in GM-CSF and IL-4. *Journal of Leukocyte Biology* 59(2):208-218.

32. Obst, R., Armandola, EA., Nijenhuis, M., Momburg, F., Hammerling, GJ. 1995. TAP polymorphism does not influence transport of peptide variants in mice and humans. *European Journal of Immunology*. Aug. 25(8):2170-2176.

33. Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. *Nature*. 375:411-415.

34. Fruh, K., K. Ahn, H. Djaballah, P. Sempe, P.M. van Endert, R. Tampe, P.A. Peterson, and Y. Yang. 1995. A viral inhibitor of peptide transporters for antigen presentation. *Nature*. 375:415-418.

35. Rock, K.L., C. Graham, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang and A.L. Goldberg. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell*. 8:761-771.

36. Harding C.V., J. France, R. Song, J,M, Farah, S. Chatterjee, M. Iqbal, and R. Siman. 1995. Novel dipeptide aldehyds are proteasome inhibitors and block the MHC class I antigen processing pathway. *J. Immunol.* 155:1767-1775.

37. Fehling H.J., W. Swat, C. Laplace, R. Kuhn, K. Rajewsky, U. Muller, and H. von Boehmer. 1992. MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science* 265:1234.

38. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in β_2-microglobulin, MHC class I proteins, and $CD8^+$ T cells. *Science.* 248:1227-1230.

39. Van Kaer, L., P.G. Ashton-Rickardt, H.L. Ploegh, and S. Tonegawa. 1992. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and $CD4^-8^+$ T cells. *Cell.* 71:1205-1214.

40. Ljunggren, H.G., L. Van Kaer, M.S. Sabatine, H. Auchincloss Jr, S. Tonegawa, and H.L. Ploegh. 1995. MHC class I expression and $CD8^+$ T cell development in TAP1/β_2-microglobulin double mutant mice. *Int. Immunol.* 7:975-984.

41. Cerundolo, V., J. Alexander, K. Anderson, C. Lamb, P. Cresswell, A. McMichael, F. Gotch, and A. Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature.* 345:449-452.

42. Lane, P., W. Gerhard, S. Hubele, A. Lanzavecchia, and F. McConnell. 1993. Expression and functional properties of mouse B7/BB1 using a fusion protein between mouse CTLA4 and human γ1. *Immunology.* 80:56-61.

43. Inaba K., Inaba M., Romani N., Aya H., Deguchi M., Ikehara S., Murumatsu S. and Steinman R.M. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176 (6):1693-1702.

44. Karre, K., H.G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature.* 319:675-678.

45. Takebe, Y., Seiki, M., Fujisawa, J-I., Hoy, P., Yokota, K., Arai, K-I., Yoshida, M. and Arai, N. 1988. Srα Promotor: an efficient and versatile Mammalian cDNA expression system composed of the simina virus 40 early promoter and the R-U5

segment of human T-cell leukemia virus type 1 long terminal repeat. *Molecular and Cellular Biology*, 466-472.

46. Zhou, X., R. Glas, F. Momburg, G.J. Hammerling, M. Jondal, and H.G. Ljunggren. 1993. TAP2-defective RMA-S cells present Sendai virus antigen to cytotoxic T lymphocytes. *Eur. J. Immunol.* 23:1796-1801.

47. Wilson, J.B., Bell, J.L., Levine, A.J. 1996. Expression of Epstein-Barr virus nuclear antigen 1 induces B-cell neoplasia in transgenic mice. *EMBO Journal*, Jun 17, 15(12):3117-3126.

48. Dildrop R.MA.A., Zimmerman, K. Hsu, E., Tesfaye, A., DePinho, R., Alt, F.W. 1989. IgH enhancer-mediated deregulation of N-myc gene expression in transgenic mice: generation of lymphoid neoplasias that lack c-myc expression. *EMBO Journal.* Apr. 8 (4):1121-1128.

Claims

1. Use of substances that impair cellular peptide processing, such as inhibitors of TAP (transporters associated with antigen processing) or of proteasome, for preparation of a pharmaceutical agent or vaccine that can stop or prevent cancer growth or virus infection by stimulating immunological effectors, especially $CD8^+$ cells, preferably cytotoxic cells, directed against antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens.

2. Use of substances according to claim 1, **characterized** in that the substances inhibit the function and/or the expression of TAP such as TAP-inhibitors e.g. ICP47 of HSV type 1, IE 12 of HSV type 2 or a gene encoding a TAP inhibitor or a nucleotide sequence that is complementary at least in part to the RNA or DNA sequences encoding TAP e.g. antisense oligonucleotides or ribozyme destroying RNA.

3. Use of substances according to claim 1, **characterized** in that the substances inhibit the function and/or the expression of proteasome such as proteasome inhibitors e.g. peptide aldehyde Z-Leu-Leu-H or a gene encoding a proteasome inhibitor or a nucleotide sequence that is complementary at least in part to the mRNA or DNA sequences encoding proteasome e.g. antisense oligonucleotides or ribozyme destroying RNA.

4. Cells, that have been treated to express antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens, to be used for preparing a pharmaceutical or vaccine against cancer or virus infections chosen from mammalian cells or non-mammalian cells e.g. cells with impaired cellular peptide processing e.g. that lack TAP and/or proteasome function and to which human MHC class I molecules have been transfected e.g. insect cells.

5. Cells according to claim 4, **characterized** in that they are mammalian cells such as hematopoetic cells, dendritic cells, preferably autologous cells, especially cells from the tissue of the origin of a cancer.

6. Lymphoid cells such as T-cells e.g. TCL, preferably $CD8^+$ T lymphocytes activated against antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens to be used for preparing a pharmaceutical or vaccine against cancer or virus infections.

7. A process for induction of antigens associated with impaired cellular peptide processing, especially MHC class I dependent antigens in mammalian cells, **characterized** in that:

a) the cells are treated with agents that inhibit substances that take place in the cellular peptide processing in mammalian cells e.g. TAP-inhibiting or proteasome inhibiting agents or

b) a sequence that codes for such an inhibiting agent is introduced into the DNA of the cell or

c) a nucleotide sequence that is complementary at least in part to the mRNA or DNA sequences encoding a substance that takes place in the cellular peptide processing in mammalian cells e.g. TAP or proteasome is introduced into the DNA of the cell or

d) the cells are treated with an appropriate ribozyme and

e) when non-mammalian cells are used, transfection thereof with human MHC class I molecules and

f) the cells may be irradiated with an appropriate dose with e.g. γ-irradiation from e.g. Cs^{137}.

8. A kit, for use in a process for induction especially of MHC class I dependent antigens associated with impaired cellular peptide processing in cells, **characterized** in that it comprises an active dose of a substance that induces MHC class I dependent antigens associated with impaired cellular peptide processing, such as an inhibitor of TAP or of proteasome or a nucleotide sequence that is complementary at least in part

to the mRNA or DNA sequences encoding proteasome or TAP e.g. antisense nucleotides or ribozyme.

9. Kit according to claim 8, further comprising appropriate adjuvants, such as e.g. cytokines and genes for costimulatory molecules, gold beads and liposomes.

10. A pharmaceutical composition or a vaccine comprising a pharmaceutically effective dose of a substance that induces MHC class I dependent antigens associated with impaired cellular peptide processing such as an inhibitor of TAP or of proteasome or a gene encoding a proteasome or a TAP inhibitor or a nucleotide sequence that is complementary at least in part to the mRNA or DNA sequences encoding proteasome or TAP e.g. antisense nucleotides or ribozyme together with a pharmaceutically acceptable adjuvant, e.g. cytokines and costimulatory molecules.

Table 1. Generation of primary CTL by Epitopes Associated with impaired TAP-function*

Effector Cells	Effector: Target ratio	Target Cells				
		TAPI -/-+	β_2m -/-	B6	RMA-S	RMA-S.TAP2
B6 anti-TAPI -/-	60:1	77[§]	22	26	46	20
	20:1	58	4	24	25	12
	7:1	41	2	18	11	8
B6 anti-RMA-S.B7-1	60:1	48	14	1	38	21
	20:1	25	10	0	20	16
	7:1	14	3	0	10	8
B6 anti-RMA-S	60:1	0	4	0	7	4
	20:1	ND[‖]	ND	ND	ND	ND
	7:1	ND	ND	ND	ND	ND

* The table shows one representative experiment out of three.

Con A blasts from TAPI -/-, β_2m -/- and B6 mice

§ Percent specific lysis

‖ Not done

Abstract

The present invention relates to use of substances that can induce expression of MHC class I dependent antigens associated with impaired cellular peptide processing for preparation of pharmaceuticals, pharmaceutical compositions or vaccines, that stimulate specific T cell mediated immune responses against cancer and virus infected cells. It also relates to mammalian cells that have been manipulated to express MHC class I dependent antigens associated with impaired cellular peptide processing and to lymphoid cells activated against such MHC class I dependent structures for the same purpose. Furthermore, processes for such manipulation of mammalian cells and kits for use in such manipulations are covered. According to the present invention, the ultimate purpose of the products or processes above is the treatment of cancers and virus infections.

Figure 1

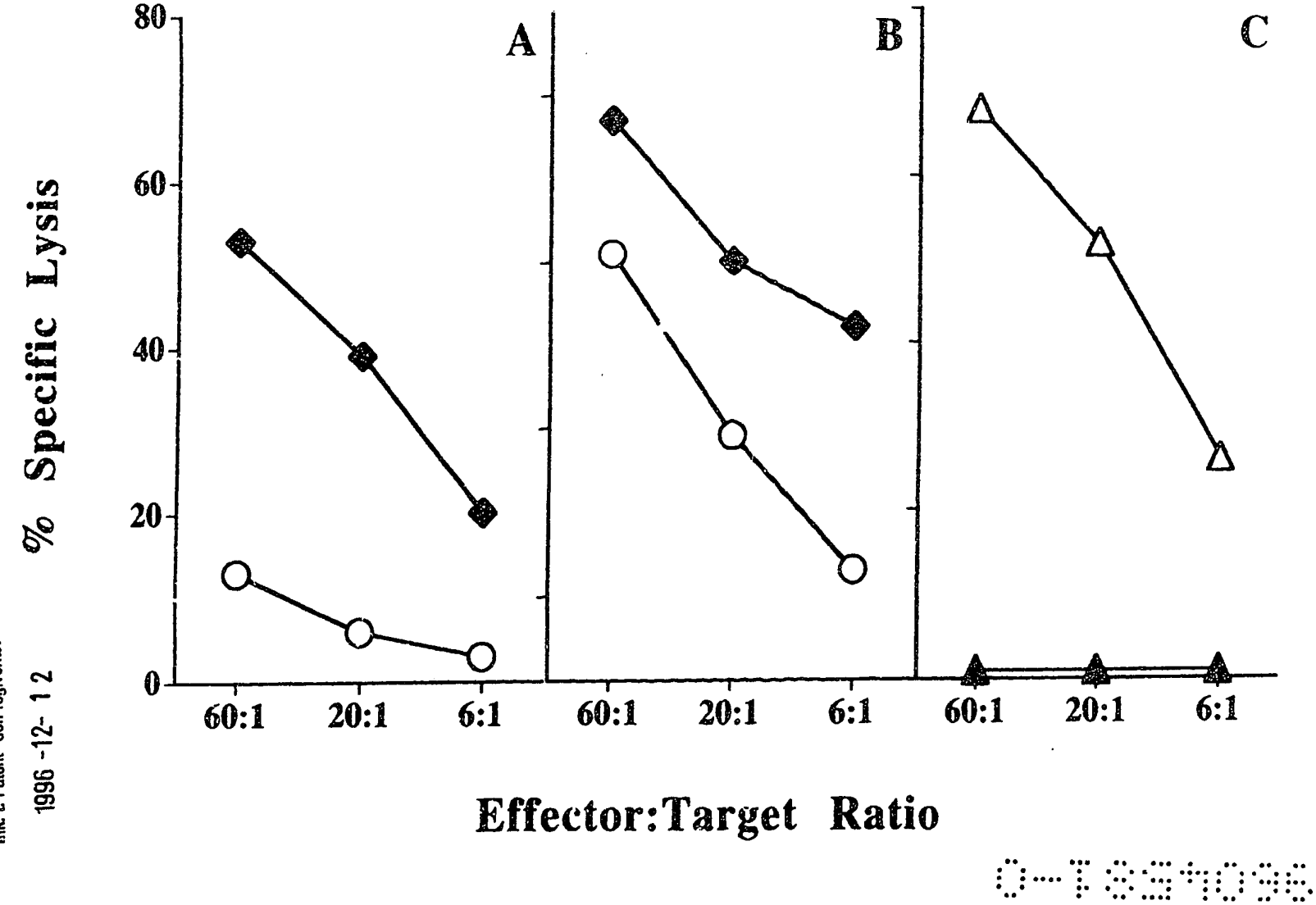


Figure 2

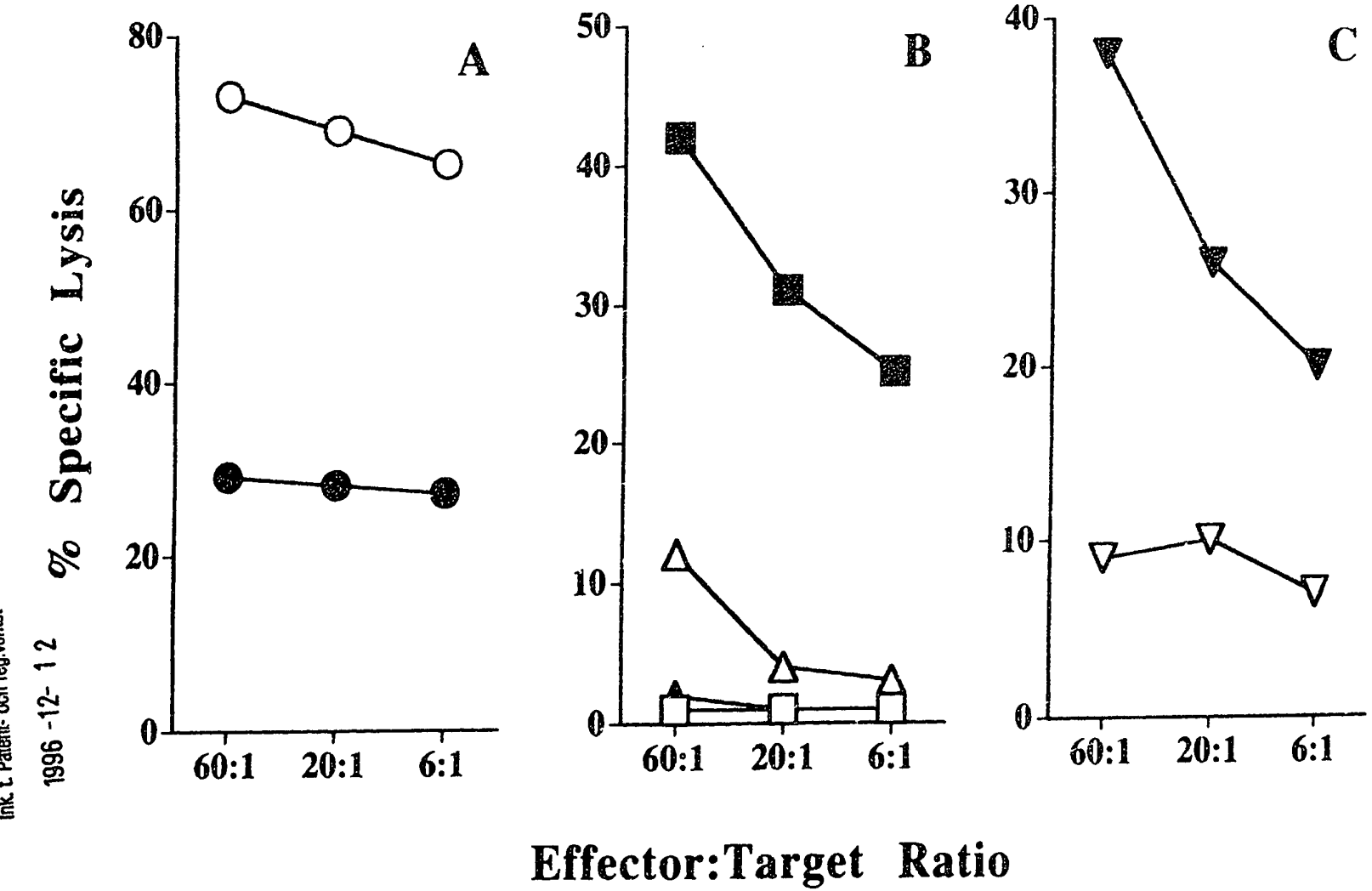


Figure 3

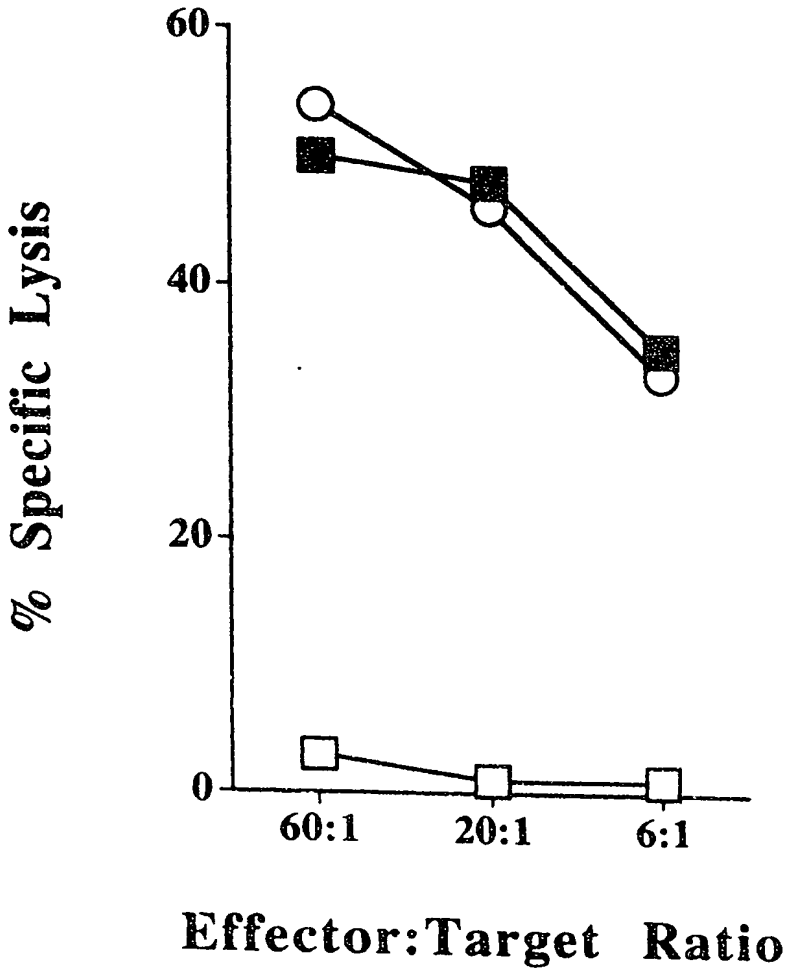


Figure 4

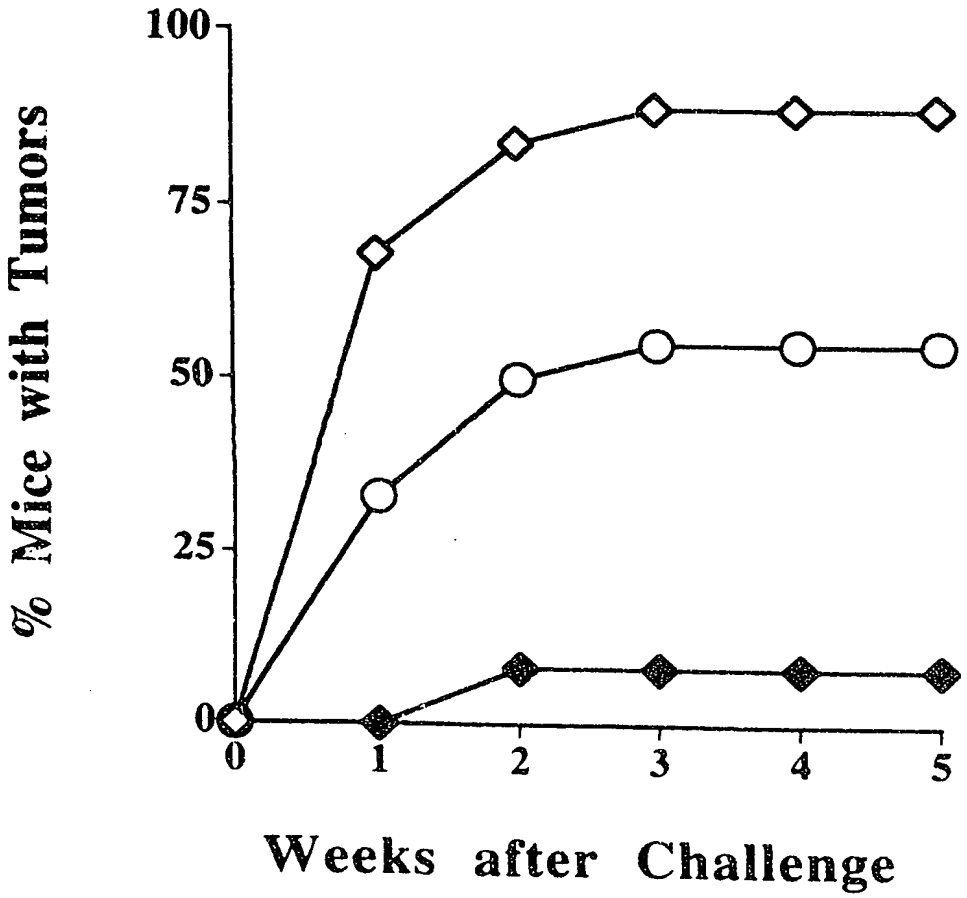


Figure 5

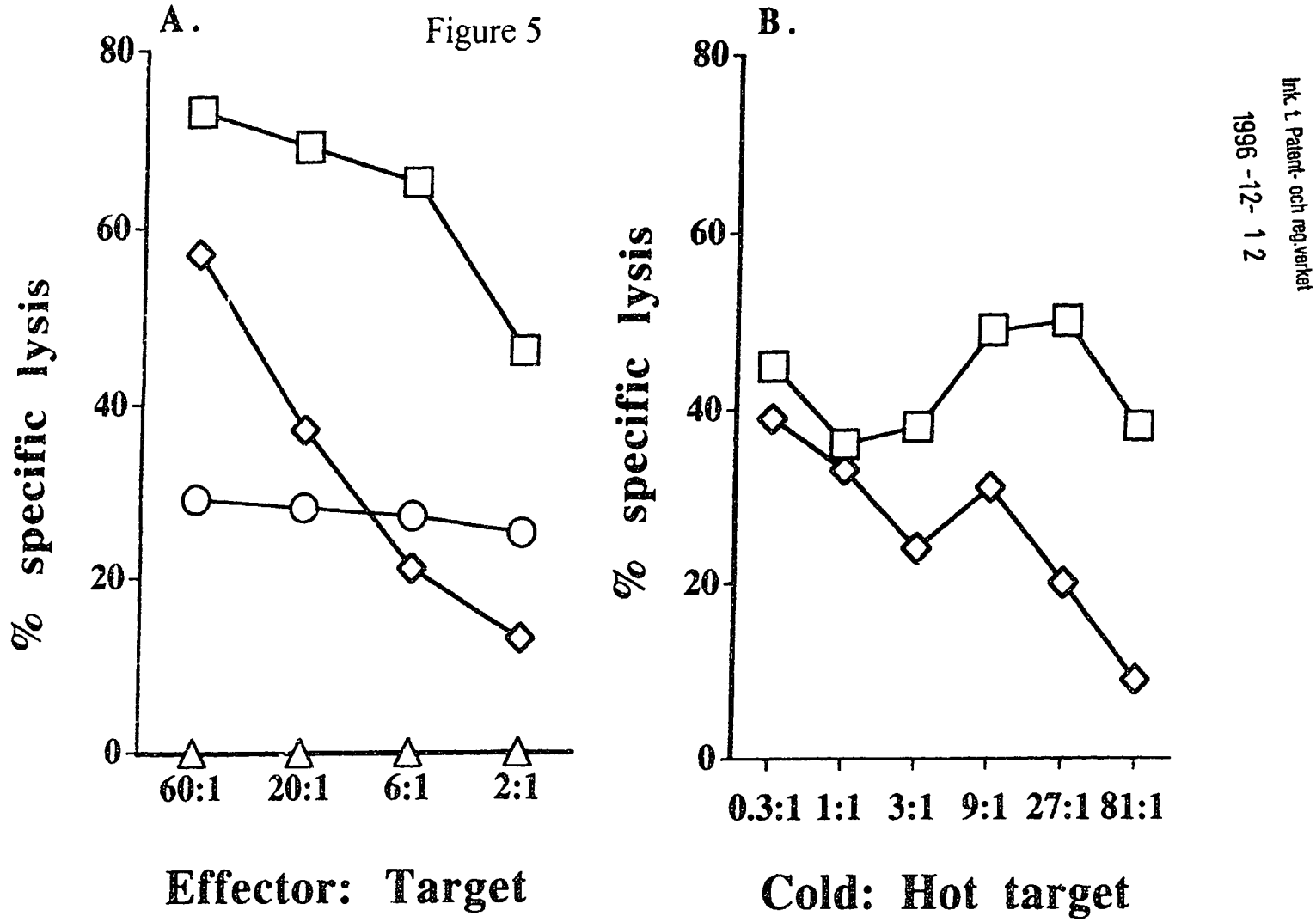


Figure 6

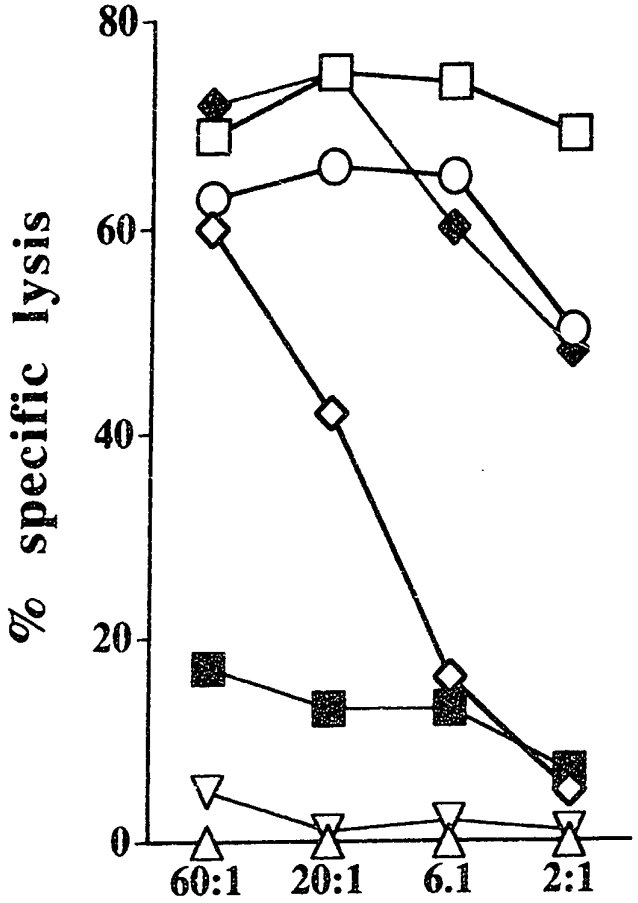


Figure 7

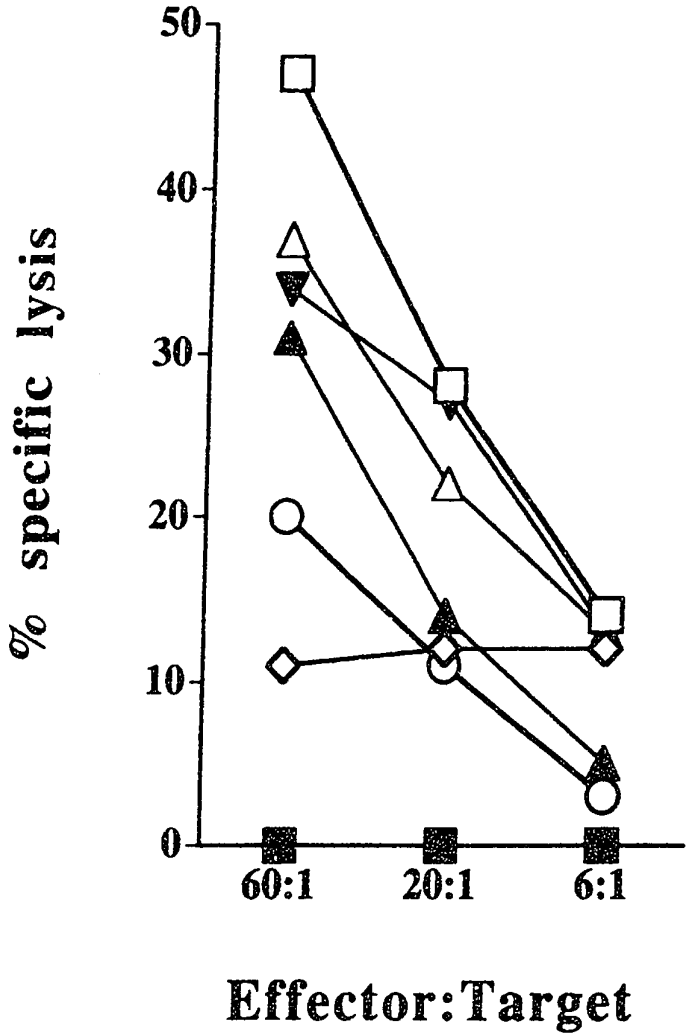
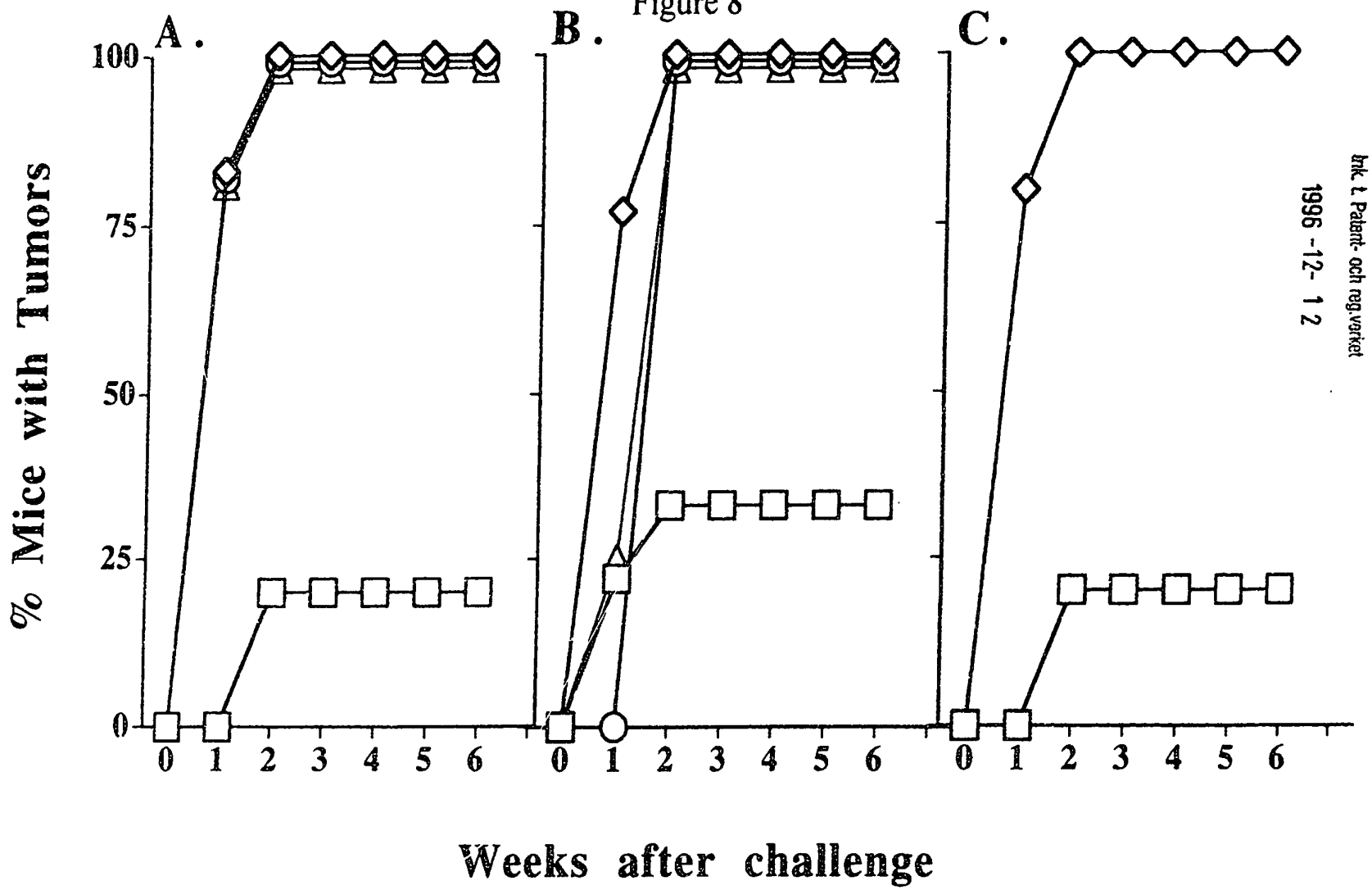


Figure 8



This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☑ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.